Algae characterization and processing techniques

Amber L. Bosley

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A Thesis

entitled

Algae Characterization and Processing Techniques

by

Amber L. Bosley

Submitted to the Graduate Faculty as partial fulfillment of the requirements
for the Master of Science Degree in Chemical Engineering

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The University of Toledo
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The concern over the increasing depletion of our nation’s fossil fuels, high oil prices and greenhouse gas emissions, has motivated research for alternative sources of energy. One alternative energy source, biofuels, provides liquid transportation fuels from biomass derived from plant or animal sources. First generation biofuels are produced from food crops abundant in sugars or lipids such as corn and soy. Second generation biofuels are produced from woody, inedible crops such as poplar and switchgrass. The third generation of biofuels is derived from algae and is of growing interest due to its high yield of energy per unit area, use of carbon dioxide for growth, and minimal contribution as a food product. The main carbon rich components of algal biomass include lipid, carbohydrates and protein. Products such as biodiesel and jet fuel can be derived from lipids. Carbohydrates, in the form of fermentable sugars, can be used to produce bioalcohols. Protein can be used as a dietary supplement or as feed for livestock.

This work addresses algal characterization and processing techniques that are helpful in utilizing algae as a feedstock for bioproduct processing. Methods for lipid analysis are compared to select a technique for small sample sizes and ease of handling. The hydrolysis of soybean oil to convert triglycerides (lipids) to free fatty acids is
evaluated. A kinetic model is developed to predict reaction behavior and serve as a platform for algal hydrolysis. Characterization techniques to determine the content of algal biopolymers; lipids, carbohydrates and protein are discussed and applied to multiple algal species. Lastly, protein extraction from alga is investigated to prepare species for successful algal hydrolysis.
I dedicate this accomplishment to all of those in my life. Every relationship has provided me encouragement and molded me into who I am today.
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Chapter 1

Thesis Introduction

A worldwide dependence on the products derived from fossil fuels has significantly increased the usage of these resources. Fossil fuels are considered to be non-renewable resources, requiring millions of years to form. This long period required to replenish these important natural resources is just one of the drivers in the hunt for alternative sources of energy. It is a U.S. national goal to develop sustainable practices for fuel production from renewable resources to reduce dependence on imported oil [1]. The United States is the top oil consumer in the world [2]. In 2009, the amount of oil produced in the U.S. was 9.14 millions of barrels per day and the amount consumed was 18.81 millions of barrels per day [2]. With the continual depletion of fossil fuels, the U.S.’s consumption rate will no longer be possible unless a viable alternative energy source is found. The increasing cost is causing difficulty for not only consumers but also businesses. Services which require the usage of fuel become more expensive to operate resulting in an increased cost to the consumer. Furthermore, fossil fuels have a high carbon footprint, which determines total greenhouse gas (GHG) emissions. Therefore, it is important that alternative energy resources have a small carbon footprint and a beneficial cost impact to the end consumer.
Alternative energy sources include wind, solar, geothermal, hydroelectric, nuclear, and biofuels. Of these, biofuels alone can provide an alternative source of liquid transportation fuels. Biofuel is obtained from carbon-rich plant and animal biomass. Plants are renewable and utilize natural sources for growth; the sun, water and carbon dioxide (a GHG). The short growth period of plants relative to fossil fuels, make them a renewable energy source. Biomass can be used as feedstock to produce various types of biofuels depending on the carbon source used such as bioalcohols, biodiesel, green diesel, bio-oil and syngas.

Biofuels are often referred to as first, second or third generation fuels. The first generation uses sugars or lipids derived from grain food crops: corn and soy being two large sources. Using these crops to produce biofuel can conflict with the food supply and pricing. The second generation of biofuels is derived from lignocellulosic biomass. These plants are non-food crops or inedible plant waste composed of the organic polymers; lignin, cellulose, and hemicelluloses. A large obstacle to overcome with woody biomass is the difficulty in accessing fermentable sugars. Lignin can impede this access. Sugars are fermented to produce ethanol using technologies similar to those in corn to ethanol production. The third generation of biofuels is produced from algae. Algae are aquatic, autotrophic organisms that chiefly contain three important biopolymers; protein, carbohydrates and lipids. Almost all algae are free of lignin. Algal species do not compete with food sources and can use waste water and carbon dioxide for growth which could be recycled from algal processing waste. In a study from 1978 to 1996, the Aquatic Species Program, investigated the use of high lipid algae for biodiesel
that could utilize waste carbon dioxide from coal fired power plants [3]. In recent years, this research and development has been revived.

The growth of algae can be more rapid than other biomass, allowing a fast turnaround for biofuel production. For most crops, the low number of harvests per year limits the yield of biofuel that can be produced. Mono-culture plots can also threaten biodiversity and can have a negative impact on greenhouse gas emissions when extensive soil fertilization is required [4]. Greenwell et al. also found that the yield of fuel per area for algae is theoretically greater than that of corn and other feedstocks, although a detailed life cycle assessment (LCA) of sustainability must be developed. A 2011 review on LCA methods concludes that there are many variables involved in the conversion of biomass to biofuels, making it difficult to make complete assessment of the viability and advantages of biofuel over fossil fuel [5]. This leads to difficulty in determining the most promising type of biomass for the replacement of transportation fuel and other energy sources. It is known that to be a practical alternative, “a biofuel should provide a net energy gain, have environmental benefits, be economically competitive, and be producible in large quantities without reducing food supplies” [6]. These factors are of utmost importance when evaluating any biomass as a potential feedstock for biofuel production.

Algae are thought to be potentially viable feedstock for fuels, motivating research and development efforts for its use in alternative energy. Biodiesel and jet fuel can be produced from lipid-rich algae. Lipid processing techniques include transesterification to convert lipids to alkyl esters (biodiesel), refining free fatty acids (FAs) or esters by catalytic cracking and hydrotreating for jet fuel production, or the UOP/Eni Ecofining™
process to obtain clean green diesel [7]. As with corn and other cellulosic biomass, carbohydrates from algae can also be used to ferment sugars for bioethanol, a bioalcohol, production. Algal proteins can be useful as livestock feed or a food supplement. Processing these important constituents utilizes the entire algal product.

Green processing of algal lipids by hydrolyzing triglycerides to FAs using high temperature (subcritical) water is the motivating goal for this study. The oil phase consisting of FAs, free of glycerol and water, can be directly converted to high quality biofuels. The use of water as a solvent eliminates the need for organic solvents and an external catalyst. The aqueous hydrolysis product containing glycerol could be a useful growth media for the algal culture, adding sustainability to processing. Protein extraction prior to lipid hydrolysis avoids charring and thermal degradation of useful proteins. Consequently, increased efficiency of hydrolysis and the ability to produce protein bioproducts can be achieved. The proposed processing scheme indicating the path to isolate FAs from lipid rich algal feedstocks is shown in Figure 1-1.

![Figure 1-1: Algal processing scheme for lipid hydrolysis to isolate free fatty acids from algae.](image_url)
In this study, Chapter 2 discusses three analytical methods used to determine the lipid/FAME content in algae. The results were compared and a direct method for converting lipids to FAMEs was chosen.

In Chapter 3, the hydrolysis of lipids in soybean oil to FAs is covered. Reaction parameters were varied and the time course of FA yield in a batch system was monitored. A kinetic model was developed to predict reaction behavior and serve as a precursor to algal hydrolysis.

Chapter 4 outlines analytical procedures used to characterize algae for lipid, carbohydrate and protein content. Results from these methods for multiple algal species are reported and discussed. This information is necessary to determine the bioproducts that each species could potentially produce.

Chapter 5 addresses methods for protein extraction from the alga *Cladophora glomerata*. Two lots of *Cladophora*, untreated and protein extracted were subjected to high temperatures, analogous to hydrolysis conditions. Charring at shorter reaction times was observed for the protein containing alga.

Chapter 6 contains final conclusions and suggestions for future work.
Chapter 2

Method Development: Comparison of FAME Analyses

2.1 Introduction

Biodiesel, derived from lipids, is commonly produced by a transesterification reaction. This reaction produces fuel as FAMEs by reacting triglycerides (a type of lipid), found in plant oil or animals fats, with methanol in the presence of an acid or base catalyst. The reaction is shown below.

\[
\text{triglyceride (TG)} \quad \overset{\text{alcohol/methanol}}{\underset{\text{alkyl esters/FAMEs}}{\leftrightarrow}} \quad \overset{\text{catalyst}}{\text{glycerol}}
\]

The current common method of lipid analysis, Bligh and Dyer extraction followed by the Napolitano method for FAME formation, involves the use of wet algae as the starting material [8, 9]. The Bligh and Dyer method is demanding of time and labor and introduces uncertainty in the dry weight analysis that must be performed separately.
Lipid content must be reported on a dry weight basis since water content of algal samples can be extremely variable.

Many algal processes or analytical techniques require the removal of water which is unavoidably collected when algae are harvested. One method of drying is lyophilization, or freeze drying. Lyophilization is used to avoid thermal degradation and to preserve sample constituents. The practice of drying algae is not ideal for most engineering applications because of high energy usage, time consumption, space limitation, and sample degradation at high temperatures. The ability to use wet algae for testing and processing is ideal to researchers and scientists, which explains the adaption of the Bligh and Dyer method. However, lipid content is calculated on a dry weight basis to maintain a universal standard, ultimately necessitating drying of samples.

The amount of time and possibilities of error make the Bligh and Dyer/Napolitano method undesirable for lab scale lipid analysis of algal species. These concerns brought focus to searching for a replacement technique. Research was done to find a simplistic way to convert algal lipids to FAMEs. In this study, three methods were tested and compared; the traditional Bligh and Dyer/Napolitano method for wet samples, requiring two steps, and two direct methods which utilize in situ transesterification of dry samples.

2.2 Methods

The following subsections contain detailed descriptions of the FAME analyses tested along with gas chromatograms and calculation methods.
2.2.1 Two-Step Method: Bligh and Dyer/Napolitano

In the Bligh and Dyer/Napolitano method (B&D/Nap), lipids are extracted and isolated then converted to FAMEs via transesterification. In the case of algal lipid extraction, this method was thought to be particularly useful to process algae with its initial water content.

In this section, the Bligh and Dyer/Napolitano (B&D/Nap) method is described in detail.

**Equipment:** A Labconco FreeZone 2.5 Liter Benchtop Freeze Dry System (Model 7670520) was used to dry algal samples. A VWR Scientific Vortex Genie 2 (VWR Scientific Products) was used for thorough mixing of samples. Cell lysing for lipid release by sonication was done using a Sonic Dismembrator equipped with an ultrasonic convertor (Model 1000, Fisher Scientific). Samples were centrifuged for the purpose of preparation and separation using an Eppendorf Centrifuge (5810R, Eppendorf) at 4000 rpm for 10 minutes at 25°C. An aluminum block DUAL Thermo Bath (ALB128, FINEPCR) was used for heating samples during reaction. A large Pyrex vacuum desiccator (3120-250, Corning) was used for drying. An Eppendorf Centrifuge (5424, Eppendorf) was used to centrifuge smaller samples.

FAME concentration was determined by means of a Shimadzu gas chromatography (GC) unit (GC-17A) equipped with a flame ionization detector (FID), an autosampler (AOC-20s) and an auto injector (AOC-20i). The software used for chromatographic peak integration was Class VP 7.4 software applications. A fused silica capillary column, Omegawax 250 with a bonded polyethylene glycol polar phase (30m x 0.25mm x 0.25μm , Restek, Cat. No. 24136), was used.
**Materials:** The following chemical reagents and solvents used for this procedure were chloroform (Fisher Scientific, Cat. No. C606SK-4), methanol (Fisher Scientific, Cat. No. A412SK-4), deionized (DI) water (U.S. Filter PURELAB Plus® High Purity Water Polishing System), toluene (Fisher Scientific, Cat. No. T324-4), 12% boron trifluoride in methanol (Acros Organics, Cat. No. 402765000) and hexane (Fisher Scientific, Cat. No. H302-4).

A suite of FAME standards were used for chromatographic peak identification. All were ordered from Nu-Chek Prep, Inc. The following were chosen as a representation of FAMEs that are expected in algal samples: methyl myristate (C14:0), methyl palmitate (C16:0), methyl stearate (C18:0), methyl arachidate (C20:0), methyl oleate (C18:1), methyl linoleate (C18:2), methyl linolenate (C18:3), methyl eicosapentaenoate (C20:5), and methyl docosahexaenoate (C22:6). An internal standard, methyl laurate (C12:0), was used to monitor concentration changes over time in samples and mixed standards.

Processing for lipid release was performed in 50 mL polypropylene sterile disposable centrifuge tubes (Fisher Scientific, Cat. No. 06-443-18). For lipid transfer and collection as well as transesterification, 10 mL Nalgene Teflon lined, screw-top centrifuge tubes (Fisher Scientific, Cat. No. 0556216C) were used. FAMEs were separated using 15 mL polypropylene sterile disposable centrifuge tubes (Fisher Scientific, Cat. No. 05-539-5). For centrifugation of the final product, 2 mL Eppendorf centrifuge tubes (Fisher Scientific, Cat. No. 05-408-138) were used. GC samples were filtered using 1 mL tuberculin slip tip syringes (B-D, Cat. No. 309602), 21 G x 1 1/2 general use sterile hypodermic needles (B-D, Cat No. 305167) and 13 mm nylon syringe
filters, 0.2 μm (Fisher brand, Cat. No. 09-720-5) to ensure the removal of suspended solids prior to injection into the GC column. Samples for the GC were prepared in 2 mL Amber screw-thread vials (Restek, Cat. No. 24621) which were capped with 8 mm screw-thread polypropylene open hole caps (Restek, Cat. No. 21177) and red polytetrafluoroethylene/silicone septa (Restek, Cat. No. 21179).

**Experimental:** The first step in the formation of FAMEs from algae in this method is the release and isolation of lipids contained in algal cells. The analysis is based on the assumption that the wet algal sample has a water content of approximately 90% on a mass basis. The ratio of chloroform, methanol and water is emphasized in the original literature for successful separation. Calculations for appropriate amounts of each solvent for wet algal samples have been done in previous work [10].

Wet algal samples are centrifuged prior to processing to pour off and remove excess water. Once the alga is concentrated into a paste, 2 g are added to 50 mL centrifuge tubes, in triplicate. Simultaneously, a portion is collected for dry weight analysis which is explained in detail in Chapter 4. This needs to be done for final calculation of FAME content on a dry weight basis. To these tubes, 2.25 mL of chloroform and 4.5 mL of methanol is added. This mixture is blended on a vortex mixer for 2 minutes followed by sonication for 2 minutes. After another 2.25 mL of chloroform is added, the mixture is again vortexed and sonicated for 2 minutes each. To achieve separation between the two layers, 1.8 mL of DI water is added, vortexed for 2 minutes and sonicated for 30 seconds. At this point, the ratio of chloroform:methanol:water is 2.25:2.25:2.025 for an algal sample with 90% water. The homogenate is then centrifuged. The empty weights of 10 mL Teflon tubes are recorded for lipid
quantification. Upon centrifugation, two layers will form; a top layer of methanol and water and a bottom layer of chloroform and lipids, separated by a conglomerate of the alga. The bottom layer is transferred to the previously weighed 10 mL Teflon lined tubes. The top layer is blended with another 2 mL of chloroform and then centrifuged again to maximize lipid extraction. The known aliquot of chloroform is dried at 40-50°C in an aluminum block thermo bath for 1-1\(\frac{1}{2}\) hours under a stream of dry nitrogen. The Teflon tubes containing dried residue are weighed to determine lipid content gravimetrically.

Lipids are then converted to FAMEs following the procedure reported in Napolitano. The dried mass of lipids is re-dissolved in 1 mL of toluene and vortexed until mixed. As a catalyst, 1 mL of boron trifluoride (10% in methanol) is added to the mixture and vortexed. Each Teflon tube is flushed with nitrogen for 30 seconds before they are capped. The solution is then heated at 100°C for a reaction time of 1 hour in the block heater. Upon removal of the samples, 2 mL of DI water is added. The tubes are then vortexed and centrifuged. The top layer consists of toluene and FAMEs and is transferred to a clean 10 mL Teflon tube using a transfer pipet. This separation is typically repeated two more times. The toluene extracts are concentrated under a stream of nitrogen and dried in a desiccator over anhydrous sodium sulfate for approximately 1 day. Once the FAME contents are dried, they are prepared for GC analysis.

### 2.2.2 Chloroform Direct Method

The Chloroform Direct Method (CDM) is an appealing alternative to the B&D/Nap method due to the one step conversion of lipids to FAMEs, in other words, \textit{in
in situ transesterification [11]. This allows for a shorter procedure time and less labor. The procedure is described in detail in this section.

**Equipment:** All equipment used is listed in Section 2.2.1.

**Materials:** 96% sulfuric acid (Acros Organics, Cat. No. 302070025) was used as a reaction catalyst. The remainder of materials required for this procedure is included in Section 2.2.1.

**Experimental:** In triplicate, 20 mg of dried alga is added to the Teflon tubes. Solvents are then added to the alga; 1.7 mL of methanol and 2 mL of chloroform. Each sample is vortexed until mixed and sonicated for 30 seconds. The solution is brought to a total volume of 4 mL by adding 0.3 mL of concentrated sulfuric acid dropwise to each sample. The tubes are vortexed briefly. The tubes are capped tightly and the weights are recorded. Samples are then placed in the block heater at 90°C for 90 minutes. The tubes are then placed in a desiccator until they cool to room temperature. The weights of the tubes are recorded to determine whether significant chloroform loss has occurred. To aid in separation, 1 mL of DI water is added and the solution is poured into 15 mL centrifuge tubes. The tubes are vortexed thoroughly for 1 minute and centrifuged. The bottom chloroform layer containing FAMEs is transferred to clean 10 mL Teflon tubes. The samples are then dried in a desiccator over anhydrous sodium sulfate under a stream of nitrogen. Once dried, FAMEs are prepared for GC analysis.

### 2.2.3 Hexane Direct Method

Another in situ transesterification method was found and tested against the B&D/Nap method and the CDM. The solvent used for the Hexane Direct Method (HDM) is hexane [12]. The details of the procedure follow.
**Equipment:** All equipment used is listed in Section 2.2.1.

**Materials:** Methanolic hydrochloric acid (Fisher Scientific, Cat. Nos. A144S-212/A412SK-4) was used as a reaction catalyst. The remainder of materials required for this procedure is included in Section 2.2.1.

**Experimental:** In triplicate, 20 mg of dried alga is added to Teflon tubes. A total volume of 2.5 mL is added to the alga: 1 mL of methanol, 0.5 mL of hexane and 1 mL of methanolic HCL. The tubes are vortexed until well mixed and weights are recorded. The samples are placed in the block heater at 100°C for 60 minutes. After completion of the reaction, the tubes are placed in a desiccator until they cool to room temperature. The weights of the tubes are recorded to determine whether significant hexane loss has occurred. For separation, 2 mL of hexane and 2 mL of DI water is added. The contents are poured into 15 mL centrifuge tubes. The samples are vortexed briefly and centrifuged. The top hexane layer is transferred to clean 10 mL Teflon tubes and dried under a stream of nitrogen over anhydrous sodium sulfate in a desiccator. Dried samples are prepared for GC analysis.

### 2.2.4 Sample Preparation

To dissolve dried FAMEs, 975 μL of hexane is added to samples and weighed. Typically, 25 μL of methyl laurate (C12:0) is also added and weighed as an internal standard. Internal standard is used for the case of sample storage as an indicator of solvent evaporation. The contents are transferred to a 2 mL Eppendorf centrifuge tube. The samples are centrifuged to separate any solids. The solution is filtered into GC vials using syringe filters to remove any suspended particles and avoid injection of solids into the GC.
### 2.2.5 Method Comparison

The main characteristics, on an average basis, between the methods are presented in tabular form below for easier comparison and discussion purposes.

**Table 2.1: Summary of the three FAME analysis methods for analysis in triplicate.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Total Amount of Alga Needed</th>
<th>Chemical Amounts</th>
<th>Catalyst</th>
<th>Time (step-by-step)</th>
<th>Total Time (approximate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bligh and Dyer / Napolitano</td>
<td>Total = 6 g wet (~300-600 mg dry)</td>
<td>Chloroform = 15 mL, Methanol = 13.5 mL, DI water = 11.4 mL, Toluene = 36 mL, Hexane = 3 mL</td>
<td>10 % BF$_3$ = 3 mL</td>
<td>Method = 7 hr, Dry Weight = 24 hr, FAME drying = 24 hr</td>
<td>55 hr ~ 2 1/2 days</td>
</tr>
<tr>
<td>Chloroform Direct Method</td>
<td>Total = 60 mg</td>
<td>Chloroform = 6 mL, Methanol = 5.1 mL, DI water = 3 mL, Hexane = 3 mL</td>
<td>Sulfuric acid = 0.9 mL</td>
<td>Method = 2.5 hr, FAME drying = 24 hr</td>
<td>26.5 hr ~ 1 day</td>
</tr>
<tr>
<td>Hexane Direct Method</td>
<td>Total = 60 mg</td>
<td>Methanol = 5.1 mL, DI water = 6 mL, Hexane = 10.5 mL</td>
<td>Methanolic HCl = 3 mL</td>
<td>Method = 2 hr, FAME drying = 24 hr</td>
<td>26 hr ~ 1 day</td>
</tr>
</tbody>
</table>

The assumptions are as follows:

- All values are representative of triplicate runs.
- For the direct methods, there is already a dried algal feedstock.
- Chemical amounts do not include standards.

### 2.2.6 External Standard Preparation

A range of external standards bordering the concentrations of FAMEs in algal samples are prepared to create calibration curves. Typically, concentrations of standards range from 0.2-5 mg standard/mL hexane. The most concentrated mixed standard is prepared first. A stock solution (C14:0-C22:6) of 100 mg standard/mL is weighed into a
GC vial. The volume of solution added is 75 µL which weighs approximately 40-45 mg. The volume is adjusted to 1500 µL by adding 825 µL (~520 mg) of hexane. The mixed standard is vortexed thoroughly. For the remaining 5-6 standards, dilution is performed by transferring 750 µL of the previously mixed standard of higher concentration into another GC vial. The combined standards are then diluted with 750 µL of hexane.

2.2.7 GC-FID Method and Operation

Gas chromatography is used as the determining method for FAME concentration. Specifications for the GC-FID unit and capillary column used can be found in Section 2.2.1. Prepared samples are placed into the auto sampler carousel, transferred to the sample rack via a robotic arm, and are injected into the column with the equipped auto injector. The column eluent undergoes pyrolysis, forming oxidized carbon ions which are then detected by a flame ionization detector. Ions are measured and displayed through the software as a chromatogram showing peak intensity per unit time. The mobile phase carrier gas is ultra high purity helium. A temperature and flow method has been developed using Class VP 7.4 software to control the GC operating parameters during run sequences. The temperature program begins at 150°C and is held for 3 minutes, then increases to 275°C at a ramp rate of 9.5°C/min where it is held for 14 minutes. The temperatures of the injector and detector are both 290°C. The total flow is 22 mL/min with a split injection ratio of 24:1 and a linear velocity of 24 cm/sec.
2.2.8 Calculations

External standard chromatograms are analyzed to form calibration curves. Below is a depiction of a typical external standard chromatogram with labels to show the location of each peak.

![Chromatogram of external FAME standards](image)

Figure 2-2: Chromatogram of external FAME standards.

The retention time is consistent for each component. The Class VP software is used to integrate the area of each standard peak for all dilutions. The mass fractions of all standards are then calculated using the weights recorded during preparation. Plots of area versus mass fraction are made for all FAMEs, resulting in nine plots. A trendline is applied to each curve using Microsoft Excel to determine the linear calibration equation. The coefficient of determination, $R^2$, is checked to ensure a good fit. The following figure is an example of a calibration plot for methyl myristate, C14:0.
Figure 2-3: Sample calibration curve for methyl myristate displaying linear regression equation (Mass Fraction = Area $*x$ + b) and coefficient of determination, $R^2$.

External standards are not only used to generate calibration curves, they also confidently estimate specific carbon chain lengths found in samples by correlating retention times based on boiling point properties of each FAME. The figure below shows two chromatograms, overlapped. The purple curve represents the external standards at 2.5 mg/mL and the blue curve depicts a product of algal lipids converted to FAMEs via transesterification.
Chromatograms for algal samples are integrated to obtain the area of each peak. Linear calibration equations are used directly for peaks that elute at the same retention time as the known standards. The area of the peak is inserted as the x variable into the linear regression equation (generic equation shown below) to calculate the mass fraction of the component.

\[
(MassFraction) = m \times (Area) + b
\]  \hspace{1cm} (2-1)

For peaks that elute at retention times between standards, theoretical mass fractions are calculated. Calibration equations for standards that border the peak are used to estimate the mass fraction by assuming upper and lower limits for carbon chain lengths.

For each individual sample, all mass fractions, \( w_i \), are summed to find a total mass fraction, \( w_T \).

\[
w_T = \sum w_i
\]  \hspace{1cm} (2-2)
The total mass of FAMEs, \( m_T \), is found by multiplying the total mass fraction and the total mass contained in the GC vial: the combined mass of FAMEs, \( m_{FAMEs} \), the GC solvent hexane, \( m_{hex} \), and the internal standard, \( m_{I.S.} \).

\[
m_T = w_T \times \sum (m_{FAMEs} + m_{hex} + m_{I.S.})
\]  

(2-3)

The FAME content in the algal sample is then calculated by dividing the total mass of FAMEs by the dry weight, \( m_{dry} \), of starting material used for the individual run.

\[
\%FAME = \left( \frac{m_T}{m_{dry}} \right) \times 100\%
\]  

(2-4)

An average of all FAME contents calculated for triplicate runs is used to represent the feedstock as a whole.

2.3 Results and Discussion

An in depth study was conducted to compare the three methods to optimize the ability to quickly, yet effectively, determine the content of FAMEs for received algal samples. Though the current Bligh and Dyer/Napolitano method is reliable and widely known, it also time consuming and labor intensive. Another concern with the B&D/Nap method was that the lipid and dry weight analysis are performed on separate samples introducing possible errors due to sample heterogeneity.

All testing was run on a frozen *Aulacoseira granulata* (diatom) stock, from 2008, after homogenization.
2.3.1 Blank Run Analysis

To determine the effects of the chemicals used in the B&D/Nap method on the materials used the procedure was run step by step, exactly as it is for lipid containing samples, sans any starting algal feedstock. This was referred to as a blank run. For a thorough investigation, a blank run was also performed for the Chloroform Direct Method. Mechanical agitation in the forms of sonication and vortexing, exposure to elevated temperatures, procedure times, and GC preparation all remained the same for the blank run. The following figures show the chromatograms for both test runs.
Figure 2-5: Gas chromatograms of blank Bligh and Dyer/Napolitano run. (a) Stand-alone of blank run. Some peaks can be seen. (b) Overlay of blank run (purple) and algal run (blue). Red circles show the location of peaks from blank run relative to FAMEs shown in sample run.
In Figure 2-5 (a), it can be seen that gas chromatography detected a few unknown peaks from the B&D/Nap blank run. The red circles in Figure 2-5 (b) indicate the overlap between FAMEs found in an algal sample and the peaks found from the blank run. Though the blank peaks are relatively small compared to FAMEs, they can still skew the calculation for FAME recovery in samples. The blank run for the CDM did not show any peaks, indicating that any results obtained from these runs are genuine to FAME content.

2.3.2 Comparison of Method Results

The three lipid converting methods were performed and compared with one another. Both of the direct methods were run with a starting sample size of 20 mg of lyophilized algae. However, a preliminary assessment on the effect of sample amount was made with the CDM by increasing the algal sample size to 100 mg. The logic behind
this test stemmed from the significant difference between the methods and the amount of feedstock used. For B&D/Nap, the dry weight equivalent to 2 grams wet alga can range considerably depending on the water content of the alga. The dry weight is generally between 100 mg to 200 mg, serving as the basis for the testing of sample amount with the CDM.

The following figures show the gas chromatograms for the three FAME methods.
Figure 2-7: Gas chromatograms of FAMEs extracted from 2008 diatom sample with the (a) Bligh and Dyer/Napolitano Method, (b) Chloroform Direct Method, and (c) Hexane Direct Method. Number peaks represent 1) C14:0; 2) C16:0; 3) C18:0; 4) C18:1; 5) C18:2; 6) C18:3; 7) C20:5; 8) C22:6
All chromatograms show the same general trend in number and intensity of peaks. The table below shows the comparison of each method and the distribution of common FAMEs found.

<table>
<thead>
<tr>
<th>FAME</th>
<th>Bligh and Dyer / Naplitano</th>
<th>Chloroform Direct Method</th>
<th>Hexane Direct Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>10 ± 0.11</td>
<td>8.3 ± 0.26</td>
<td>7.4 ± 0.42</td>
</tr>
<tr>
<td>C16:0</td>
<td>20 ± 0.50</td>
<td>14 ± 0.75</td>
<td>23 ± 2.3</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.0 ± 0.10</td>
<td>2.0 ± 0.15</td>
<td>1.6 ± 0.26</td>
</tr>
<tr>
<td>C18:1</td>
<td>0.60 ± 0.02</td>
<td>1.9 ± 0.16</td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td>C18:2</td>
<td>1.2 ± 0.01</td>
<td>2.1 ± 0.08</td>
<td>1.5 ± 0.18</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.82 ± 0.01</td>
<td>1.9 ± 0.11</td>
<td>0.74 ± 0.01</td>
</tr>
<tr>
<td>C20:5</td>
<td>4.4 ± 0.07</td>
<td>4.7 ± 0.10</td>
<td>4.0 ± 0.45</td>
</tr>
<tr>
<td>C22:6</td>
<td>2.0 ± 0.09</td>
<td>3.2 ± 0.27</td>
<td>3.6 ± 0.72</td>
</tr>
</tbody>
</table>

There is good agreement of the recovery of individual FAMEs between all three methods. The calculated FAME content for each experiment is shown in Table 2.3. Figure 2-8 shows method results graphically.
Table 2.3: Results from FAME methods showing the content of converted lipids in 2008 diatom samples.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Dry Weight (mg)</th>
<th>FAME Recovery (mg)</th>
<th>% FAME</th>
<th>Average % FAME Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform Direct Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.7</td>
<td>1.8</td>
<td>8.6</td>
<td></td>
<td>9.6 ± 0.82%</td>
</tr>
<tr>
<td>21.1</td>
<td>2.1</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.7</td>
<td>2.2</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.8</td>
<td>1.9</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform Direct Method 100 mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99.4</td>
<td>8.8</td>
<td>8.9</td>
<td></td>
<td>7.7 ± 1.22%</td>
</tr>
<tr>
<td>104</td>
<td>8.0</td>
<td>7.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>94.2</td>
<td>6.1</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexane Direct Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.8</td>
<td>1.2</td>
<td>4.6</td>
<td></td>
<td>4.3 ± 0.73%</td>
</tr>
<tr>
<td>23.7</td>
<td>1.2</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.9</td>
<td>0.7</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bligh &amp; Dyer / Napolitano</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>149</td>
<td>10</td>
<td>7.0</td>
<td></td>
<td>6.7 ± 0.34%*</td>
</tr>
<tr>
<td>148</td>
<td>9.3</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>146</td>
<td>9.5</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>149</td>
<td>10</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* based on an average dry weight

Figure 2-8: FAME recovery from three methods. The sample size was varied for the first (chloroform/methanol) dry method.
It can be seen from this data that all methods produced similar FAME recoveries. The CDM produced the highest amount of FAMEs, with a small standard deviation. The HDM resulted in the lowest yield of FAMEs.

To determine whether analysis results were statistically different, an analysis of variance (ANOVA) was performed on samples. Based on the p-value of 4.8E-05 obtained from the ANOVA, we can say that there is a significant difference in lipid content between methods. The ANOVA was followed by a Fisher multiple comparison test. The HDM method yielded significantly lower lipid content. The lipid content as measured by the B&D/Nap method and the CDM (using 100 mg sample size) was not significantly different within a 95% confidence interval. The CDM method with a 20 milligram (mg) sample size did produce significantly higher results than the 100 mg sample size. However, reproducibility of lipid measurement with the 20 mg sample size was good with a standard deviation of less than 10% of the measured value.

The effect of dry weight analysis on the variability of B&D/Nap results was investigated. It can be seen in Table 2.3 that the B&D/Nap FAME conversion was based on an average dry weight. The effect of calculated dry weight was tested by separately lyophilizing four portions of the homogenized 2008 diatoms to obtain four dry weights. Though the samples were from the same source, one of the tubes showed a much larger dry weight percent than the other three. This is shown below in Table 2.4 summarizing the FAME results for the multiple dry weight percentages found.
Table 2.4: Effect of measured dry weight % on FAME recovery

<table>
<thead>
<tr>
<th>Tube</th>
<th>Dry Weight %</th>
<th>Wet Weight %</th>
<th>% FAME Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.23</td>
<td>90.8</td>
<td>5.26</td>
</tr>
<tr>
<td>2</td>
<td>6.66</td>
<td>93.3</td>
<td>7.29</td>
</tr>
<tr>
<td>3</td>
<td>6.53</td>
<td>93.5</td>
<td>7.43</td>
</tr>
<tr>
<td>4</td>
<td>6.68</td>
<td>93.2</td>
<td>7.27</td>
</tr>
<tr>
<td>Average</td>
<td>7.28</td>
<td>92.7</td>
<td>6.67</td>
</tr>
</tbody>
</table>

These results pose a problem because we need completely homogeneous samples in order to have confidence in our results from a wet algal sample. Homogeneity is difficult to achieve with wet algae. Another issue is that dry weight percent is calculated separately from the sample used in the experiment. Though a conscious effort is made to collect a sample for drying near the area that the experimental sample is taken, it is seen that this may not be a fair representation of dry weight for the alga used in the method. While it is ideal to use a method that does not require prior drying of the alga, it is not effective if the results are dependent on dry weight calculations which can be inconsistent. It also requires extra alga consumption (experiment and dry weight determination) which is problematic when algal supplies are limited. In contrast, the dry methods are not biased by dry weight analysis because the starting material is already dry.

2.4 Conclusions

Table 2.1 clearly shows the distinct differences between methods. Perhaps the most important advantage to the direct methods is the significantly less amount of alga needed to run the transesterification reaction. The CDM is less labor and time intensive compared to Bligh and Dyer/Napolitano. Another major difference is that the B&D/Nap
method requires almost five times the amount of reagents used for the whole procedure. Although chloroform is still used as a solvent, and is not ideal to work with, the catalyst is easier to use than the HDM. In summary, the CDM requires less feedstock, time, labor and material costs while providing relatively quick means to determine the FAME content in algal samples. This method has been chosen for FAME analysis of all subsequent samples.
Chapter 3

Hydrolysis of Glycerides from Soybean Oil in Subcritical Water: A Kinetic Study

3.1 Introduction

It is difficult and energy intensive to ensure complete removal of water from algal feedstocks. To overcome the challenges in processing a lipid feedstock with high water content, a two-step, green processing technique using subcritical water (SBW) for lipid hydrolysis has been examined. The drying of algal species can be omitted when hydrothermal processing is used [13]. Conversion of lipids (largely triglycerides) to free fatty acids (FAs) yields a product of low melting point and greatly reduced vapor pressure, providing a platform for fuel production.

In the hydrolysis or hydrothermal liquefaction of lipid-rich biomass, triglycerides react with water to form FAs which is easily separated from the aqueous phase after cooling. This reaction is often acid or base catalyzed.

SBW is used not only as a reactant, but a solvent and catalyst as well. At temperatures between its boiling point (100°C) and critical point (374°C), liquid water is in its subcritical state with properties approaching that of organic solvents. As temperature increases, the dielectric constant of SBW decreases, improving the solubility
of the oil (glycerides) in the aqueous phase. Upon completion of the lipid hydrolysis, glycerol, which has high water solubility, can be recycled back with the water stream to the algal culture to increase the production capacity of the growth media. The FA phase can then be esterified to FAMES, used as feedstock for catalytic cracking and conversion to jet fuel, or processed in other routes.

The hydrolysis of lipids to FAs in subcritical water has been studied using soybean oil as a model system to guide future kinetic studies of algal lipid hydrolysis. As eukaryotic organisms, algae are more complex than soybean oil, with significant protein and carbohydrate content. In an environment of high temperature and pressure, charring of algal components is a concern. The complex structure of algae and its constituents could also limit mass transfer of reactants or products, slowing overall reaction rates. A hydrolysis kinetic model has been developed for the less complex soybean oil / SBW system. Once a reliable reaction model is established, algae, containing high water content, can then be processed directly as received.

### 3.2 Soybean Oil Hydrolysis

Batch hydrolysis studies were completed using a 300 mL Parr reactor. Processing parameters such as reaction temperature and time, agitation speed, and final product extraction technique were varied. Intermittent samples were collected and analyzed for FA content. The FA yield (YFA) at multiple time points and temperatures was used to obtain estimates of the kinetic model parameters.
3.2.1 Chemicals

The soybean oil used for hydrolysis was purchased through Sigma Aldrich (Cat. No. S7381-1L). For complete extraction of the FAs from the aqueous phase, diethyl ether (Fisher Scientific, Cat. No. E134-1) and sodium chloride (Fisher Scientific, Cat. No. S271-1) are used [14]. For GC analysis, FAs are dissolved in chloroform (Fisher Scientific, Cat. No. C606SK-4) for FA determination and hexane (Fisher Scientific, Cat. No. H302-4) for glyceride analysis.

External standards are prepared to quantify FA concentration. The following were purchased through Nu-Chek Prep, Inc: myristic acid (C14:0, N-14A), palmitic acid (C16:0, N-16A), stearic acid (C18:0, N-18A), oleic acid (C18:1, U-46A), linoleic acid (C18:2, U-59A) and linolenic acid (C18:3, U-62A). The selection of standards were based on FA distribution in soybean oil [10, 15]. Heptadecanoic acid is used as an internal standard (C17:0, N-17A).

A set of premixed standards are used to determine the approximate retention times of glycerides. The standard solutions were purchased through DCG Partnership I, Ltd (Complete Kit Part #: D6584 Table 2 Kit with Internal Standards Added). The standards include glycererin, monoolein, diolein, triolein, butanetriol, and tricaprin.

3.2.2 Reactor System and Sampling

The time course of the soybean oil hydrolysis is followed using a lab-scale batch system with a Parr reactor model 4560 (Parr Instrument Company). The 300 mL reactor vessel is constructed of T316 stainless steel (452HC). To ease clean-up, a borosilicate glass liner is used (762HC). The system is equipped with a controller (4843) to monitor temperature and agitation speed. The removable reactor head contains an agitator.
(stirrer), a thermocouple, sampling tube and an internal cooling coil. The agitator is driven by a light duty magnetic drive. Water for the internal cooling coil is supplied by a circulating cool water bath (Model 1156, VWR Scientific Products). For rapid reactant delivery, a pressure bomb from Parr (304L-HDF4-150) is used. The lab-scale Parr reactor is shown in Figure 3.1.

![Parr reactor system. Key operation components are labeled. The reactor head and parts are shown in smaller picture.](image)

The batch experiments were previously set at a reactant ratio of oil:water of \( \sim 1:4 \) [14]. Water, \( 120 \pm 3 \, \text{g} \), is weighed and poured into the reactor glass liner. The liner is placed into the steel reactor vessel and securely attached to the reactor head. The reactor
heating jacket is placed around the vessel. Using the controller, the temperature and agitation are set to the desired values. For temperatures between 225-300°C, 45-120 minutes can be required to stabilize temperature. For this reason, a pressure bomb was implemented to act as a holding vessel for the oil reactant (30 ± 3 g of soybean oil). Once the reaction temperature is stable, ±1°C, the bomb is charged with nitrogen gas at a pressure ~50 psi higher than the reaction vessel. The valve to the nitrogen cylinder is then closed and the valve on the bomb outlet is opened, discharging contents of the bomb rapidly into the reactor vessel. Once the soybean oil is transferred, the valve is closed. At chosen time intervals the reactor contents (2-5 mL) are collected into glass test tubes by opening the sampling tube valve for periodic sampling of the FA product. At the completion of the reaction sampling, the heating jacket is removed, and the water flow to the cooling coil from cooling bath is started. The temperature typically drops to 100°C within 5-8 minutes. When cool, the reactor is disassembled and the glass liner is removed. The contents are poured into a separatory funnel or Erlenmeyer flask.

3.2.3 Sample Preparation

At higher temperatures, the oil phase can form an emulsion, causing a cloudy or milky white aqueous phase and difficulty in separating the oil and water phases (Figure 3-2).
Figure 3-2: Water/oil mixture final product (left) and intermittent samples (right) from a hydrolysis reaction. The aqueous layer is noticeably cloudy/milky. An emulsion can be seen in a number of intermittent samples.

To separate the phases, a small amount of sodium chloride is added to each test tube (~0.1 g) and final reactor contents (~1.5 g). The samples are thoroughly mixed. Diethyl ether is then added to all samples; 1 mL for intermittent samples and 15 mL to the final product. The samples are mixed well. Time is allowed for the oil and water phases to clear (Figure 3-3).

Figure 3-3: Final product (left) and an intermittent sample (right) after extraction. The aqueous layer is transparent and emulsions are broken.

3.2.4 Chromatographic Analysis

Analysis of FAs as a function of time and the distribution of glycerides in soybean oil is carried out via GC using a Shimadzu gas chromatography unit (GC-17A) equipped with a flame ionization detector (FID). Samples are injected into the GC by an
autosampler (AOC-20s) and an auto injector (AIC-20i). Class VP 7.4 software is used for GC method, sequence and data analysis. A Stabilwax®-DA GC column (30m x 0.53mm x 0.25μm, Restek, Cat. No. 11025) with a crossbond carbowax – polyethylene glycol stationary phase was used to separate FAs. For analyzing total glycerides in soybean oil, an MXT®-Biodiesel TG column (15m x 0.32mm x 0.10μm, Restek, Cat. No. 70293) with Siltek® treated stainless steel was used. Details of GC operation can be found in Chapter 2.

After extraction, the oil layer is removed and transferred to a clean test tube once a clear aqueous phase is observed. The ether is allowed to evaporate. The oil samples are prepared for GC analysis of FAs by dissolving 75 μL (~55 mg) of oil into 950 μL of chloroform (~1.3 g). Internal standard is used to monitor the changes in the sample over time of storage, due to solvent evaporation.

The GC temperature program for FA analysis begins at 40°C and increases to 160°C at a ramp rate of 6°C/min, followed by a second temperature ramp of 15°C/min to 250°C where it is held for 25 minutes. The temperatures of the injector and detector are both 280°C. The total flow of helium carrier gas is 182 mL/min with a split injection ratio of 25:1 and a linear velocity of 45 cm/sec.

To analyze the total glyceride content of soybean oil, ~75 mg of oil is dissolved in ~550 mg of hexane. Serial dilution is performed until 5-6 soybean oil samples have been prepared. Multiple GC samples are run to guarantee good peak resolution.

For the determination of total glyceride content in soybean oil, the GC oven program is as follows: 60°C for 1 minute, 20°C/min to 180°C, 15°C/min to 230°C, 40°C/min to 400°C, hold for 7 minutes, 40°C/min to 420°C, final hold for 10 minutes.
The injector and detector temperatures are both 340°C. The total flow of the helium carrier gas is 279 mL/min. The linear velocity is 50 cm/sec linear velocity and a split ratio of 100:1 is used.

External FA calibration standards (5-6) are prepared for GC analysis using C14:0, C16:0, C18:0, C18:1, C18:2 and C18:3 standards. Typically, concentrations of standards range from 0.25 to 6 mg standard/mL of chloroform.

As described in Chapter 2, the areas of the peaks for each standard are used to develop calibration curves and obtain linear calibration equations for peak area versus mass fraction of FA in the sample. The FA chain length/identification is approximated by comparison of sample and standard retention times. FAs of longer alkyl chain length exhibit longer retention times.

Some of the FAs show possible trans-cis isomerization, resulting in a peak doublet for some of the FA alkyl chain lengths, especially at higher reaction temperatures [14]. In these cases, the mass fraction of the isomers is estimated using the calibration equation for the standard of the nearest retention time. The figure below shows a typical chromatogram for the final product from the hydrolysis reaction compared to a calibration standard.
Figure 3-4: Gas chromatogram showing the overlay of a hydrolysis sample (blue) and a calibration standard (purple).

The individual mass fractions of each FA, $w_j$, are summed to find a total mass fraction, $w_T$, of FAs in the GC sample, then used to find the total mass, in mg, of FAs in each sample.

$$w_T = \sum w_j \quad (3-1)$$

$$m_T = w_T \times \sum (m_{PA} + m_{CDB} + m_{S.S.}) \quad (3-2)$$

The FA analyzed by weight fraction is converted to mole fraction using the average molecular weight of FAs from the measured distribution of carbon chain length found in soybean oil. The measured distribution of FAs in soybean oil (Table 3.1) is in close agreement with that reported by others [15].
Table 3.1: The distribution of FAs in soybean oil for products from three hydrolysis reaction temperatures compared to the distribution found in van Gerpen.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>van Gerpen (wt.%)</th>
<th>Measured (wt.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250°C</td>
<td>275°C</td>
</tr>
<tr>
<td>C16:0</td>
<td>6-10</td>
<td>11</td>
</tr>
<tr>
<td>C18:0</td>
<td>2-5</td>
<td>4.6</td>
</tr>
<tr>
<td>C18:1</td>
<td>20-30</td>
<td>23</td>
</tr>
<tr>
<td>C18:2</td>
<td>50-60</td>
<td>52</td>
</tr>
<tr>
<td>C18:3</td>
<td>5-11</td>
<td>8.8</td>
</tr>
</tbody>
</table>

The average molecular weight of FAs was calculated as 278 g/mol based on Table 3.1.

The theoretical concentration of FA, CFA, is found from the distribution of glycerides in soybean oil. As measured by GC, it is found that the oil is composed of only triglycerides (TG). Diglycerides (DG) were detected via GC, but in trace amounts which were too small to quantify. The molar yield of FA, YFA, is calculated from CFA in oil sample analyzed by GC divided by theoretical CFA from the amount of glycerides in the starting soybean oil.

\[
YFA = \frac{CFA_{\text{experimental}}}{CFA_{\text{theoretical}}} \quad (3-3)
\]

3.3 Results and Discussion

The effect of agitator speed on observed soybean oil hydrolysis rate was assessed by varying the speed from 300 to 700 rpm at 250°C, and ~750 psig. Observed reaction
kinetics at an agitation speed of 700 rpm appeared to be free of mass transfer limitations. The repeatability and effects of extraction were tested at 250°C. Using the agitation speed of 700 rpm, a range of temperatures was tested: 225, 250, 275 and 300°C. Good repeatability and high YFA was reached at all reaction temperatures with the exception of 225°C. A kinetic model was developed with kinetic parameters to fit the experimental data.

### 3.3.1 Agitation Speed

Agitator speed was varied at 300, 500 and 700 rpm to determine speeds required to avoid reaction with mass transfer limitation between aqueous and oil phases. Water is a reactant with incomplete miscibility between TG and water.

The observed reaction rate is expected to be a function of the intrinsic reaction rate when mass transfer between phases is rapid. There are three typical cases of the observed reaction rates. For slow reaction rate and rapid mass transfer, the observed reaction rate constant, \( k_{\text{obs}} \), is equal to the intrinsic rate constant, \( k_r \). Alternatively, a rapid reaction rate could be accompanied by slow mass transfer, which would be a mass transfer limited reaction where the observed rate constant is equal to the overall mass transfer coefficient, \( k_c \). When the reaction is not limited by reaction rate or mass transfer, the observed rate constant is a function of both intrinsic reaction rate and mass transfer constants. It is desirable to obtain reaction rate data in the regime where mass transfer is rapid and intrinsic reaction rates are observed [16].

The Frössling correlation provides a functional relationship between the Sherwood number (dimensionless mass transfer coefficient) and the Reynolds and
Schmidt numbers (Equation 3-4) for dispersed particles (or in this case oil) in a continuous phase.

\[ Sh = 2 + 0.6 Re^{1/2} Sc^{1/3} \]  \hspace{1cm} (3-4)

Where,

\[ Sh = \frac{k_c d_p}{D_{AB}} \]  \hspace{1cm} (3-5)

\[ Re = \frac{\rho d_p U}{\mu} \]  \hspace{1cm} (3-6)

\[ Sc = \frac{\mu}{\rho D_{AB}} \]  \hspace{1cm} (3-7)

\[ k_c = \text{mass transfer coefficient (m/s)} \]
\[ d_p = \text{diameter of pellet (m)} \]
\[ D_{AB} = \text{diffusivity of component A in B (m}^2\text{/s)} \]
\[ \rho = \text{fluid density (kg/m}^3\text{)} \]
\[ U = \text{free-stream velocity (m/s)} \]
\[ \mu = \text{fluid viscosity (kg/m/s)} \]

Fluid velocity increases with agitator speed resulting in an increase in the Reynolds number and a concomitant increase in the Sherwood number. With high mass transfer rates between aqueous and oil phases, intrinsic reaction kinetics are observed and increasing fluid velocity has no effect on observed reaction rates. YFA is used as indication of the effect of fluid velocity on observed reaction kinetics. YFA versus batch reaction time are plotted for varying agitator speed (Figure 3-5).
Figure 3-5: FA yield versus time for varied agitator speeds 300, 500 and 700 rpm. Data at 700 rpm were collected in triplicate with error bars shown for one standard deviation. Data at 300 and 500 rpm are from single experimental runs. Lines are drawn to show the trends of FA formation over the course of the hydrolysis reaction.

At 300 rpm, samples collected prior to a 40 minute batch reaction time did not contain sufficient oil fraction for analysis, indicating a non-homogenous reactor solution and poor mixing. For this reason, data points at 20 and 30 minutes are not reported for 300 rpm. Yields at 300 rpm were lower compared to agitation speeds of 500 and 700 rpm. YFA at 500 rpm were often slightly greater than or approximately equal to those at 700 rpm, indicating that the reaction is not mass transfer limited between these two agitator speeds. An agitator speed of 700 rpm was chosen for further experiments to ensure high mass transfer between aqueous and oil phases.
3.3.2 Repeatability and Extraction

Subcritical water hydrolysis of soybean oil was run at 250°C, ~750 psi, and 700 rpm to test the repeatability of hydrolysis results. Repeated runs were also used to compare ether/salt extracted oil samples with non-extracted samples. When the reaction temperature was increased above 250°C, emulsions began to appear, necessitating addition of an extraction step. In Figure 3-6, good agreement in YFA versus time is seen for repeated runs with and without ether extraction.

![Figure 3-6: Non-extracted (red) vs. extracted (black) hydrolysis samples at 250°C.](image)

Non-extracted samples show good repeatability at low YFA and exhibit increased deviation as time and YFA increases. Extracted samples show good repeatability over the entire reaction range of YFA. This may be due to better consistency in FA recovery from the aqueous phase for extracted samples.
Residual ether in extracted samples was determined by GC analysis. The mass fraction of the ether in samples was within the range of $2 \times 10^{-4}$ to $4 \times 10^{-4}$ for the data sets in Figure 3-6. The minimal fraction of ether suggests nearly complete evaporation of the solvent prior to analysis.

3.3.3 Temperature Effects

Reaction temperatures tested were 225, 250, 275 and 300°C. YFA as a function of time at each temperature is shown in Figure 3-7.
(b) YFA vs. Time (min) for Run 1, 250°C and Run 2, 250°C.

(c) YFA vs. Time (min) for Run 1, 275°C, Run 2, 275°C, and Run 3, 275°C.
Hydrolysis data at reaction temperatures 250, 275 and 300°C appear consistent between runs. At 225°C, YFA are low and data appear less consistent. At the lower reaction temperature of 225°C, samples drawn from the bottom of the reactor vessel through the reactor sampling tube appeared to have little of the oil phase. The oil phase appears less soluble in subcritical water at the lower temperature of 225°C possibly introducing mass transfer limitations between reactant water and TG. The dielectric constant of subcritical water decreases in a non-linear manner as temperature increases [17]. Between the reaction temperatures of 225 to 300°C water exhibits dielectric properties similar to that of acetonitrile, methanol, ethanol and acetone (with increasing temperature) [18, 19]. Literature sources confirm that solubility of soybean oil and fatty acids increases as the dielectric constant of the solvent decreases [18, 20]. Due to data inconsistencies and low YFA at 225°C, these data were not included in kinetic modeling.
3.3.4 Kinetic Modeling

Data at reaction temperatures 250, 275, and 300°C were used to develop a reaction kinetic model. This model will be used as framework in the planning of future algal hydrolysis experiments.

The hydrolysis of TG, DG and MG (monoglycerides) to their respective glycerides and FAs can be modeled as a set of three reversible reactions shown below.

\[
\begin{align*}
\text{TG} + \text{W} & \rightleftharpoons_{k_1}^{k_3} \text{DG} + \text{FA} \\
\text{DG} + \text{W} & \rightleftharpoons_{k_1}^{k_3} \text{MG} + \text{FA} \\
\text{MG} + \text{W} & \rightleftharpoons_{k_1}^{k_3} \text{G} + \text{FA}
\end{align*}
\]

TG, DG, and MG are hydrolyzed to form a glyceride or glycerol and one mole of FA in equations 3-8 to 3-10. Kinetic constants for hydrolysis were assumed to be the same for these three groups of glycerides (TG, DG and MG) and independent of the carbon chain length of the fatty acid groups. These assumptions reduce the number of number of specific reaction rate constants from six to two.

Previously reported specific reaction rate constants for sunflower oil hydrolysis in SBW using the six reaction rate constants for equations 3-8 to 3-10 did not exhibit the sigmoidal trend in YFA versus time seen in experimental data in Figure 3-7 [21]. The sigmoidal behavior of YFA versus time in the batch data suggests an autocatalytic phenomenon [22, 23]. FAs can serve as an acid catalyst for the hydrolysis of glycerides
Autocatalyzed hydrolysis can produce increased reaction rates without the addition of an external catalyst. In addition to equations (3-8 to 3-10), the autocatalytic reversible reactions have been added to the reaction model.

\[
\begin{align*}
TG + W + FA & \xrightarrow{k_2/k_4} DG + 2FA \quad (3-11) \\
DG + W + FA & \xrightarrow{k_2/k_4} MG + 2FA \quad (3-12) \\
MG + W + FA & \xrightarrow{k_2/k_4} G + 2FA \quad (3-13)
\end{align*}
\]

The kinetic rate constants, \(k_i\), were assumed to exhibit an Arrhenius dependency upon temperature.

\[
k_i = 10^{a_i} \exp \left( \frac{-E_i}{RT} \right) \quad (3-14)
\]

For primary reactions, \(i = 1\) (forward), 3 (backward)
For autocatalytic reactions; \(i = 2\) (forward), 4 (backward)

Pre-exponential factors \((a_i)\) and activation energies \((E_i, \text{kJ/mol})\) for the hydrolysis/esterification of ethyl oleate/oleic acid were taken from recent literature as a reasonable starting point to begin modeling for soybean oil hydrolysis [22]. The reaction rate expressions were assumed to be proportional to the stoichiometric coefficients in equations 3-8 to 3-13. These reactions are actually quite complex involving many elementary steps including dissociation, protonation, hydrolysis, and deprotonation by
FA catalysts [23]. The following ordinary differential equations (ODEs) were developed, representing the empirical mole species balances for each component for batch hydrolysis of soybean oil.

\[
\frac{d(TG)}{dt} = -k_1(TG)(W) + k_3(DG)(FA) - k_2(TG)(W)(FA) + k_4(DG)(FA)^2
\]  
(3-15)

\[
\frac{d(DG)}{dt} = -k_3(DG)(FA) - k_1(DG)(W) + k_1(TG)(W) + k_3(MG)(FA) - k_4(DG)(FA)^2 - k_2(DG)(W)(FA) + k_2(TG)(W)(FA) + k_4(MG)(FA)^2
\]  
(3-16)

\[
\frac{d(MG)}{dt} = -k_3(MG)(FA) - k_1(MG)(W) + k_1(DG)(W) + k_3(G)(FA) - k_4(MG)(FA)^2 - k_2(MG)(W)(FA) + k_2(DG)(W)(FA) + k_4(G)(FA)^2
\]  
(3-17)

\[
\frac{d(G)}{dt} = -k_3(G)(FA) + k_1(MG)(W) - k_4(G)(FA)^2 + k_2(MG)(W)(FA)
\]  
(3-18)

\[
\frac{d(W)}{dt} = -k_1(CTG)(W) + k_3(CG)(FA) - k_2(CTG)(W)(FA) + k_4(CG)(FA)^2
\]  
(3-19)

\[
\frac{d(FA)}{dt} = -k_3(CG)(FA) + k_1(CTG)(W) + k_2(CTG)(W)(FA) - k_4(CG)(FA)^2
\]  
(3-20)

Where,

\[
(CTG) = (TG) + (DG) + (MG)
\]  
(3-21)

\[
(CG) = (DG) + (MG) + (G)
\]  
(3-22)

Initial molar concentrations (mol/L) of reaction components at time zero (t=0) were calculated using the quantities of oil (30 g) and water (120 g) fed to the reactor. The concentration of TG was calculated to be 0.226 mol/L from the distribution of glycerides in the starting soybean oil found via GC and using an average molecular
weight of TG based on the estimated FA molecular weight. The molar concentration of 120 g of water is 43.7 mol/L.

A simple Euler method could not be used to solve the ODEs and deviated from a fourth-order Runge-Kutta method. The figure below shows YFA versus time predicted by the Euler and Runge-Kutta methods.

![Figure 3-8: Comparison of the FA yield versus time models developed using the Euler and Runge-Kutta methods.](image)

The change of concentrations over time \((dC_i/dT)\) for all components (TG, DG, MG, G, W and FA) was found using the Runge-Kutta method to integrate the ordinary differential species balances. In this method the ODE function is solved by evaluating four points of the derivative over a time step, \(h\): once at the initial point, twice at two midpoints and once at the endpoint. The four points are combined to solve for the full time step. The Runge-Kutta equations are as follows.
RK in the equations represents Runge-Kutta to distinguish between the numerical method equations and rate constants contained in the ordinary differential equations.

These equations were applied for all time steps to find the respective concentrations of each species as a function of time. The equilibrium constant for both sets of reactions (primary and catalyzed) should be equal, resulting in equations 3-28 and 3-29 [22]. The number of kinetic parameters, $a_i$ and $E_i$, the specific reaction rate prefactor and activation energy, respectively, then reduces from eight to six.

$$a_3 = a_1 - a_2 + a_4 \quad (3-28)$$

$$E_3 = E_1 - E_2 + E_4 \quad (3-29)$$

The kinetic parameters of the reaction model were regressed using the Solver function of Excel to minimize the sum of squared errors between molar concentrations from experiment and model.
A single set of parameters were found for the entire range of temperatures. The values of the pre-exponential factors and activation energies as well as the kinetic rate constants are shown below.

Table 3.2: Kinetic parameters found for soybean oil hydrolysis from the fourth-order Runge-Kutta method.

<table>
<thead>
<tr>
<th>$k_i$</th>
<th>$a_i$</th>
<th>$E_i$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.05</td>
<td>98.5</td>
</tr>
<tr>
<td>2</td>
<td>2.12</td>
<td>45.3</td>
</tr>
<tr>
<td>3</td>
<td>10.8</td>
<td>135</td>
</tr>
<tr>
<td>4</td>
<td>6.92</td>
<td>81.4</td>
</tr>
</tbody>
</table>

Table 3.3: Kinetic rate constants for three hydrolysis reaction temperatures.

<table>
<thead>
<tr>
<th>$k_i^*$</th>
<th>250°C</th>
<th>275°C</th>
<th>300°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>1.64E-04</td>
<td>4.61E-04</td>
<td>1.18E-03</td>
</tr>
<tr>
<td>$k_2$</td>
<td>3.98E-03</td>
<td>6.39E-03</td>
<td>9.85E-03</td>
</tr>
<tr>
<td>$k_3$</td>
<td>2.51E-03</td>
<td>1.03E-02</td>
<td>3.74E-02</td>
</tr>
<tr>
<td>$k_4$</td>
<td>6.09E-02</td>
<td>1.43E-01</td>
<td>3.12E-01</td>
</tr>
</tbody>
</table>

* $k_1$ and $k_3$ units are (mole/L)$^{-1}$(min)$^{-1}$; $k_2$ and $k_4$ units are (mole/L)$^{-2}$(min)$^{-1}$.

The activation energies for each reaction can be used to estimate the heat of reaction, $\Delta H^\circ_{rxn}$, which can be calculated using measured heats of formation at a reference temperature (typically 298K), $\Delta H^\circ_f$. This serves as a means to evaluate the validity of kinetic parameters and compare experimental results with thermodynamic predictions.
The van’t Hoff equation relates the equilibrium constant to the overall heat of reaction,

\[
\frac{d \ln k_{eq}}{dT} = \frac{\Delta H^o_{\text{rxn}}}{RT^2}
\]  

(3-30)

The equilibrium constants for each set of reactions, primary and catalyst, are equivalent to one another. Therefore, the following relationship can be established.

\[
k_{eq,\text{forward}} = \frac{k_1}{k_3} = k_{eq,\text{reverse}} = \frac{k_2}{k_4}
\]  

(3-31)

This equation can be verified for the specific reactions using the kinetic rate constants determined from the reaction model. Inserting one of the equilibrium constant equations into equation 3-31 and integrating, the following relationship is developed [24].

\[
k = Ae^{-\Delta H/RT}
\]  

(3-32)

Relating equation 3-32 to the Arrhenius equation (equation 3-14), it is found that the difference in activation energies is equal to the overall heat of reaction.

\[
\Delta E = E_{\text{forward}} - E_{\text{reverse}} = \Delta H^o_{\text{rxn}}
\]  

(3-33)

The heat of reaction is calculated from the following overall reaction.

\[
\text{TG} + 3\text{W} \xleftrightarrow[k_3]{k_1} \text{G} + 3\text{FA}
\]  

(3-34)

The overall heat of reaction is calculated to be -66 kJ/mol of TG using formation enthalpies from NIST (Table 3.4) for TG, triolein, and FA, oleic acid (C18:1) [25].
Table 3.4: Heat of formations used for estimating the heat of reaction of soybean oil.

<table>
<thead>
<tr>
<th>Component</th>
<th>$\Delta H_f^{298}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triolein</td>
<td>-2194</td>
</tr>
<tr>
<td>Water</td>
<td>-285</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-670</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>-815</td>
</tr>
</tbody>
</table>

The difference between the forward and reverse activation energies found from the kinetic model is -36 kJ/mol of TG. Considering the simplifying assumptions in the kinetic model and in the calculation of the heat of formation using only C18:1 rather than the fatty acid distribution, the two values correspond well.

The pre-exponential factors and activation energies are used to formulate rate constants for each reaction temperature. Figure 3-9 shows the experimental data (in symbols) for 250, 275, and 300°C compared to the kinetic model (lines) generated by the kinetic model.

Figure 3-9: Soybean oil hydrolysis kinetic model (lines) compared to experimental data (symbols) for varied reaction temperatures.
There is good agreement of YFA between with experimental data and that predicted using the kinetic model. This is further demonstrated by a parity plot (Figure 3-10), which compares experimentally measured FA yield to that predicted by the kinetic model.

Figure 3-10: Parity plot for model vs. experimental hydrolysis data.

There are no systematic deviations between experimental and predicted concentrations. In addition, the concentration of all hydrolysis components versus time can be found in Appendix A.

### 3.4 Conclusions

A series of batch experiments for SBW hydrolysis of soybean oil were completed. Agitation speeds of 300, 500 and 700 rpm were investigated to ascertain mixing conditions to avoid mass transfer limited reaction. A speed of 700 rpm was found to be sufficient for effective mass transfer. Effect of reaction temperature was studied at 225,
250, 275 and 300°C. The reaction showed good repeatability for the three higher temperatures but low YFA and poor repeatability for 225°C. A kinetic model was developed for the remaining temperatures using a fourth-order Runge-Kutta numerical method to solve the ODEs comprising the mole species balances for the batch reactor data. The model shows a good fit and will be used for future experimental planning for algal hydrolysis. The effects of varying starting conditions can be tested by the model before the adjustments are made to the experimental system. The robustness of the model can be evaluated. The autocatalytic behavior of FA suggests green methods for enhanced lipid hydrolysis with partial recycling of reaction products (FA) to reactor feed.
Chapter 4

Compositional Screening of Algal Samples

4.1 Introduction

When considering algae for alternative energy purposes, it is important to devise an efficient and economical processing strategy for the biomass. It is ideal to use all possible components of algae with minimal waste to offset the concerns that are associated with some of the proposed methods of algal collection and processing.

In order to consider all possible products from algae, it is critical to understand the constituents of the biomass. This is done by initial screening for the main biopolymers contained in algal samples; lipids, carbohydrates and protein.

The methods discussed in this chapter are dry weight, lipid/FAME, carbohydrate (cellulose and starch), and protein analyses. Those species collected from Lake Erie and screened were Cladophora glomerata (green alga), Aulacoseira granulata (diatoms) and diatoms of an unknown species. Multiple harvests of green alga from varied growing conditions were received from the photobioreactor (PBR) and screened. These species are Scenedesmus dimorphus, and Chlorella vulgaris.
4.2 Compositional Analyses

The following subsections give detailed descriptions of the analyses used to determine the characteristics of algal samples, specifically the composition of biopolymers.

4.2.1 Dry Weight Analysis

The moisture content of algal species is significant in preparing for processing techniques. It is particularly important in processes tolerant of wet starting material to determine the initial ratio of alga:water and decide if an adjustment is necessary.

**Equipment:** Labconco FreeZone 2.5 Liter Benchtop Freeze Dry System, Model 7670520.

**Experimental:** Room temperature algal samples are added to 50 mL centrifuge tubes, enough to coat the conical bottom of the tubes. The weights are recorded of the alga plus the tubes. The samples are frozen then attached to the lyophilizer where they are subjected to vacuum to remove the water content. Once the samples are dried to a constant weight, they are removed from the lyophilizer. The final weights at room temperature are recorded.

**Calculations:** To calculate the dry weight percent of a sample, $dry\%$, the final dry weight, $m_f$, after freeze drying is divided by the initial wet weight, $m_i$.

$$dry\% = \left( \frac{m_f}{m_i} \right) \times 100\%$$  \hspace{1cm} (4-1)
When it is useful to know the amount of water present in an algal species, the wet weight percent, wet%, can be found by subtracting the dry weight percent from 100%.

\[
\text{wet}\% = 100\% - \text{dry}\%
\]  

(4-2)

It is highly recommended to perform dry weight analysis on multiple samples to obtain an average when a sufficient amount of alga is available. As seen in Chapter 2, the dry weight can heavily vary within a lot of alga.

4.2.2 Lipid/FAME Analysis

The conversion of algal lipids, mainly triglycerides, to FAMEs yields information on the quantity of lipids and carbon chain lengths. It is a highly significant analysis for alga that is intended for fuel production. The FAME analysis used for all received algal samples is described in Section 2.2.2.

4.2.3 Carbohydrate Analysis

Important carbohydrates are present in algae as starch and cellulose. Starch is an energy storage carbohydrate while cellulose is a structural component found in the cell walls of plants. The repeating unit of these biopolymers is glucose. The difference between the two is the glucose linkages that bond the units; α-1-4-linkages in starch and β-1-4-linkages in cellulose. Glucose can be used to produce bioethanol. Quantification of glucose determines the starch and cellulose content by the following methods.
4.2.3.1 \textit{Structural Carbohydrate and Lignin Analysis}

The procedure used to detect cellulose as well as lignin content is reported in the National Renewable Energy Laboratory (NREL) analytical procedure “Structural Carbohydrate and Lignin Analysis” [26]. In this procedure, a two-step acid hydrolysis is performed, breaking cellulose into its glucose monomers which are easier quantified through high performance liquid chromatography (HPLC). The total glucose found by this method will also include the glucose that is contributed by starch. To distinguish between the two, starch analysis must be performed separately, as discussed in the next section.

Lignin is separated into acid soluble lignin and acid insoluble lignin, which also contains any ash present in the biomass. To date, lignin has only been found in marine, red algae, while other algae appear to lack lignin [27]. When new algal species are received, they are still tested for lignin and ash, though it is not expected that such components are present.

\textbf{Materials:} Chemicals used in the structural carbohydrate analysis are as follows: 72\% sulfuric acid (RICCA, Cat. No. R8191600-2.5D), and calcium carbonate (Fisher Scientific, Cat. No. C64-500). From Acros Organics, alpha-D(+)-glucose (AC17008-0010), D(+)-mannose (AC15060-0250), L(+)-arabinose (AC10498-0250) and D(+)-cellobiose (AC10846-0250). From Sigma-Aldrich, D-(+)-xylose (X1500-500G) and D-(+)-galactose (G0750-25G).

Along with standards, a control sample is run with the rest of the samples being analyzed. This control is one in which the amounts of structural carbohydrates and lignin
are known. Poplar, provided by NREL, is used as a positive control and compositional analysis was compared to published results [28].

Table 4.1: Comparison of measured and expected amounts of structural carbohydrates found in the poplar control.

<table>
<thead>
<tr>
<th>Carbohydrates, %</th>
<th>Expected</th>
<th>Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>37</td>
<td>35</td>
</tr>
<tr>
<td>Xylan</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Galactan</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Arabinan</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Mannan</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

The sugars are quantified using HPLC with refractive index detection on the Aminex HPX-87P carbohydrate analysis column. The mobile phase used for this column is filtered DI water at a flow rate of 0.6 ml/min. The column temperature is held constant at 80°C.

4.2.3.2 Starch Analysis

The assay used for starch analysis uses enzymatic hydrolysis to fractionate starch into glucose monomers using thermostable alpha-amylase and amyloglucosidase in two phases (Megazyme, # K-TSTA). In phase one, starch is solubilized and partially hydrolyzed by thermostable alpha-amylase. In phase two, amyloglucosidase is used to completely hydrolyze starch dextrins to glucose which can then be quantitatively detected via HPLC.

Materials: For the starch assay, materials not included in the Megazyme kit were ethanol (A406F-1GAL) and sodium acetate (BP334-1), both from Fisher Scientific.
**Experimental:** The modified total starch assay protocol from Megazyme is performed on algal samples as well as a control maize standard. In triplicate, 100 mg of sample is added to a glass test tube. For dispersion, 0.2 mL of 80% v/v ethanol is added to each tube. Thermostable alpha-amylase is diluted 1:30 with sodium acetate buffer (100 mM, pH 5.0). This diluted solution, 3 mL, is added to each tube, mixed and placed into the aluminum block heater at 100°C for 6 minutes. After partial hydrolysis, 0.1 mL of amyloglucosidase is added to the tubes and heated at 50°C for 30 minutes. For samples that contain a lower concentration of starch (0-10%), the content of the tube is added to a 10 mL volumetric flask. The volume is adjusted with DI water. For the maize standards and samples that contain higher starch content (10-100%), the solution is diluted 1:100. The samples are then centrifuged in 2 mL centrifuge tubes. The contents are added to HPLC vials using a syringe with a filter.

The samples and glucose standards are analyzed for glucose by an HPLC equipped with a refractive index detector and an Aminex HPX-87H carbohydrate analysis column. The mobile phase used is HPLC grade 5mM sulfuric acid at a flow rate of 0.6 ml/min with a column temperature of 65°C.

The starch content is determined as glucan by the same procedure used in the NREL carbohydrate analysis. The percentage of starch contained in the maize standard is compared to expected results. If the starch content is as expected, it can be concluded that enzymes are active and the assay was run properly.

4.2.4 **Protein Analysis**

Protein content is significant to the introduction of algae to the lab-scale Parr reactor for hydrolysis. This is discussed in more detail in Chapter 5. It is also important
to determine if the main product from an algal species will most likely be derived from protein.

An elemental analysis which measures carbon, hydrogen and nitrogen (CHN Elemental Analysis (Perkin Elmer 2400 Series II CHN Elemental Analyzer)) is used to quantify protein content found in algal samples. Combustion analysis is used to obtain the masses of combustion products of these elements, which are then used to calculate the elemental composition of the sample. A protein factor of 4.44 is used to convert the amount of nitrogen into an estimate of protein content [29].

**Experimental:** Dry samples are weighed in triplicate into tin capsules in quantities within the range of 3-5 mg. The samples are then run through the CHN analyzer along with acetanilide as a standard. The standard is used to check the accuracy of analysis. The tin capsules are injected into a combustion chamber in an excess of oxygen. Here, carbon dioxide, water, and nitric oxide are formed. The combustion products are then sent through a chromatography column by helium carrier gas. The eluent is detected by a thermal conductivity detector and the elemental composition is measured.

**Calculations:** CHN results report carbon, hydrogen, and nitrogen in weight percent. Once the results are received, the nitrogen content found for the samples is extracted. The weight percent of nitrogen, $w_N$, is multiplied by a factor of 4.44 to calculate an estimated weight percent of protein, $w_p$.

$$w_p = w_N \times 4.44 \quad (4-3)$$

Although CHN analysis measures total nitrogen, the protein factor was developed for microalgal biomass, giving a good estimate of protein content.
4.3 Results

Algal samples were subjected to one or more of the discussed processing methods. Not all species were thoroughly investigated. The interest for the algae varied between lipid/FAME content for hydrolysis processing, protein content for the purpose of protein extraction experiments (discussed in Chapter 5), and carbohydrate content for species which may be ideal for total consumption of algae based on supply and possible products.

The tables below summarize the results obtained from all analyses on a species basis. Table 4.2 shows the percent of dry weight, FAMEs and protein found in all species. Carbohydrate content was found for two species of *Scenedesmus* received from the PBR (Table 4.3).
Table 4.2: Results from multiple analyses for algal samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>Month/Year</th>
<th>Source</th>
<th>Dry Weight %</th>
<th>Lipid/FAME %</th>
<th>Protein %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladophora Glomerata</td>
<td>09/2009</td>
<td>Lake Erie</td>
<td>23</td>
<td>1.7 ± 0.21</td>
<td>13 ± 0.91</td>
</tr>
<tr>
<td>Cladophora Glomerata</td>
<td>07/2010</td>
<td>Lake Erie</td>
<td></td>
<td></td>
<td>9.3 ± 0.44</td>
</tr>
<tr>
<td>Aulacoseira granulata</td>
<td>2008</td>
<td>Lake Erie</td>
<td>9.6</td>
<td>7.7 ± 1.2</td>
<td>13 ± 0.36</td>
</tr>
<tr>
<td>Diatoms (species unknown)</td>
<td>06/2010</td>
<td>Lake Erie</td>
<td>5.1</td>
<td>2.5 ± 1.4</td>
<td>18 ± 0.18</td>
</tr>
<tr>
<td>Scenedesmus dimorphus</td>
<td>2010</td>
<td>Covered Pond</td>
<td></td>
<td>5.2 ± 0.77</td>
<td>33 ± 0.67</td>
</tr>
<tr>
<td>Scenedesmus dimorphus</td>
<td>05/2010</td>
<td>PBR</td>
<td>13</td>
<td>19 ± 3.0</td>
<td>10 ± 0.34</td>
</tr>
<tr>
<td>Scenedesmus dimorphus</td>
<td>07/2010</td>
<td>PBR</td>
<td>9.3</td>
<td>5.4 ± 0.69</td>
<td>30 ± 0.21</td>
</tr>
<tr>
<td>Scenedesmus dimorphus</td>
<td>09/2010</td>
<td>PBR</td>
<td>28</td>
<td>25 ± 1.2</td>
<td>8.1 ± 0.05</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>02/2011</td>
<td>PBR</td>
<td>64</td>
<td>9.4 ± 1.7</td>
<td>18</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>04/2011</td>
<td>PBR</td>
<td>68</td>
<td>21 ± 2.3</td>
<td>7.8 ± 0.05</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>05/05/2011</td>
<td>PBR</td>
<td>66</td>
<td>14 ± 0.32</td>
<td>13 ± 0.08</td>
</tr>
<tr>
<td>Chlorella vulgaris</td>
<td>05/25/2011</td>
<td>PBR</td>
<td>87</td>
<td>15 ± 0.78</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 4.3: Sugar analyses for algal samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>Starch %</th>
<th>Carbohydrates %</th>
<th>Structural Glucan %</th>
<th>Total Lignin/Ash %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scenedesmus dimorphus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05/2010 PBR</td>
<td>5.9 ± 0.4</td>
<td>Glucan: 13, 12</td>
<td>7</td>
<td>0 / 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xylan: 0.49, 0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galactan: 0.45, 0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arabinan: 0.10, 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mannan: 2.7, 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scenedesmus dimorphus</td>
<td>12 ± 0.1</td>
<td>Glucan: 36 ± 1.4</td>
<td>24</td>
<td>0/0</td>
</tr>
<tr>
<td>09/2010 PBR</td>
<td></td>
<td>Xylan: 0.95 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Galactan: 0.83 ± 0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arabinan: 0.12 ± 0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mannan: 3.11 ± 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.4 Discussion

The results discussed in this section can be referenced in Tables 4.2 and 4.3. Each species will be discussed separately.

4.4.1 Cladophora glomerata

Cladophora glomerata was collected from Lake Erie in 2009 and 2010. There is information that suggests Cladophora glomerata from the Great Lakes can act as a harbor for enteric bacteria by helping its growth and release into the surrounding water source, making the analysis of this species important for human health [30]. The FAME content measured in 2009 was low at 1.7%, which is consistent with a reporting in
literature of 5% [31]. The moderate protein content found in both stocks made the species ideal for preliminary protein extraction experiments, discussed in Chapter 5.

### 4.4.2 Diatoms

Analyses were performed on two stocks of diatoms. The frozen stock from 2008 was used to validate lipid extraction techniques as discussed in Chapter 2. The lot collected in 2010 was found to have a low amount of lipids, 5.1%, but significant amount of protein.

### 4.4.3 *Scenedesmus dimorphus*

*Scenedesmus* was received from two different sources. The lot in summer 2010 was provided by research partners, Algaeventure, and had been grown in an open pond. The species was not strictly monitored allowing for susceptibility to contamination or invasion of other species. The low lipid content and high protein content could be an indication of this.

The other three lots from May, August and September of 2010 were grown in the PBR. The species from 07/2010 had low lipid content and high protein content. It was found that rotifers had invaded the species, most likely contributing to increased protein content. The other two lots had a significant amount of lipids, 13 and 28%. The range expected for *Scenedesmus dimorphus* is between 16 and 40% lipids [32].

*Scenedesmus* from the photobioreactor from 05/2010 and 09/2010 were taken through analysis to determine carbohydrate content. The results can be found in Table 4.3. Based on results, the alga could be quite useful for other biofuel applications.
Starch analysis was also performed on the *Scenedesmus* species from 05/2010 and 09/2010. Starch analysis distinguishes between the amount of structural glucan and that contributed by starch. When considering processing techniques for entire algal stocks, it is important to know the full carbon content of the species to determine the potential yield of useful bioproducts.

### 4.4.4 *Chlorella vulgaris*

In 2011, *Chlorella vulgaris* was grown in the PBR. It is seen in Table 4.2 that the PBR harvest from 02/2011 has a significantly higher dry weight, as do the remainder of PBR algae after that date. At that time, the PBR site implemented a continuous feed centrifuge from SRS (SR 1020). This centrifuge has a processing capability of 396 gal/hr with a maximum centrifugal force of 10,350 x g.

It is reported that *Chlorella vulgaris* usually contains 14-22% lipids [32]. During the growth of *Chlorella*, experiments studying the effect of nitrogen on lipid content were performed for each lot. The nitrogen was provided as NaNO$_3$. The concentration of this chemical was varied between each harvest. The tables below show the amount nutrients and nitrogen concentration for each lot.
Table 4.4: Total list of nutrients for all algal lots.

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Macronutrients (g)</th>
<th>Micronutrients (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>147</td>
<td>Na₂EDTA</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>148</td>
<td>FeCl₃·6H₂O</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>50</td>
<td>MnCl₂</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>35</td>
<td>ZnSO₄</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>varied</td>
<td>NaMO₄</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CoCl₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CuSO₄</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₃BO₃</td>
</tr>
</tbody>
</table>

Table 4.5: Nitrogen concentration for *Chlorella vulgaris* growth experiments.

<table>
<thead>
<tr>
<th>Species</th>
<th>NaNO₃ (g)</th>
<th>Concentration (mM)</th>
<th>Lipid/FAME %</th>
<th>Protein %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella vulgaris</em> 02/2011 PBR</td>
<td>340</td>
<td>1.08</td>
<td>9.4 ± 1.7</td>
<td>18</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em> 04/2011 PBR</td>
<td>3.4</td>
<td>0.01</td>
<td>21 ± 2.3</td>
<td>7.8 ± 0.05</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em> 05/05/2011 PBR</td>
<td>7</td>
<td>0.02</td>
<td>14 ± 0.32</td>
<td>13 ± 0.08</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em> 05/25/2011 PBR</td>
<td>15.25</td>
<td>0.05</td>
<td>15 ± 0.78</td>
<td>10</td>
</tr>
</tbody>
</table>
The general trend shows that the algal species from 04/2011, 05/05/2011, and 05/25/2011 all have higher lipid content and lower protein content than the sample from 02/2011. Though more data would need to be collected to form a definite conclusion, the experiments show what may be expected. It is hypothesized that lipid content will decrease with increased nitrogen starvation. Growth nutrients added to the PBR will continue to be adjusted in an attempt to produce a species of optimal lipid content.

4.5 Conclusions

Dry weight analysis is highly variable due to the various collection methods for each species. This can be seen by the increase of dry weight percent when a powerful, continuous feed centrifuge was implemented at the PBR.

FAME content is essential for algal samples being considered for oil recovery via hydrolysis. If a species does not have high lipid content, hydrolysis in the Parr reactor may not be beneficial.

Carbohydrate and protein analysis give indication as to which species may need processing prior to hydrolysis. Currently, the focus is on protein content as the effect of charring may be caused by denatured protein. Carbohydrate content will be examined before and after hydrolysis to determine if removal is necessary.

While receiving samples from the PBR, it is important to have structured methods to determine the composition of the algal samples. This will give PRB operators an indication of the effects of the nutrient levels used to grow the culture, allowing for adjustments to be made to the nutrient levels for the next culture.
Chapter 5

Algal Protein Extraction

5.1 Introduction

In Chapter 3, the hydrolysis of soybean oil to access free fatty acids from glycerides was discussed. These experiments are precursors for the addition of algae to the Parr reactor. The final goal is to process algal samples under subcritical conditions to isolate FAs from other algal components. Separation of components such as proteins may allow processing of this product stream without degradation that may occur at FA processing conditions.

At high temperatures used in lipid hydrolysis, protein is vulnerable to thermal denaturation and charring. Various protein extraction methods were assessed. Exposures of alga, pre and post extraction, to high temperatures were made to determine the effects of subcritical conditions on algal samples. This chapter discusses the observations found from these preliminary tests as well as detailed protein extraction procedures and respective results.
5.2 Extraction Procedures

A number of scholarly journal articles were found on the topic of protein extraction in algae [33-36] and soy flakes [37]. In testing extraction techniques, importance was placed on finding a successful procedure to be used universally on many algal species that has a short processing time. All tests are described in detail and were run in triplicate. A short summary and comparison of all procedures can be found in Table 5.1.

5.2.1 Materials

Feedstock: For the first set of single extractions, a frozen stock of *Cladophora glomerata* from the 2009 collection in Lake Erie was available at the time extractions were carried out. *Cladophora glomerata*, collected from Lake Erie in July 2010, was used for all other extractions.

Materials: The chemicals used for the extraction methods, ordered from Fisher Scientific, are as follows: sodium hydroxide pellets (Cat. No. S320-1), 2-hydroxy-1-ethanethiol, 99% (β-mercaptoethanol) (Cat. No. AC12547-0100) and hydrogen peroxide (H$_2$O$_2$), 3% (Cat. No. H312-500).

Analysis: Upon separation of the aqueous, protein phase from the alga, the supernatants were frozen and the algal pellets were freeze-dried. CHN analysis was used to determine the nitrogen content remaining in the pellets [33]. The details of this analysis can be found in Chapter 4. To calculate estimated protein content, the nitrogen content is multiplied by a protein factor of 4.44.
5.2.2 Single Extraction at High pH

The first procedure tested was adapted from a method for protein extraction from soy flakes by Karki _et al._ in 2010. This method was tested due to its short extraction times and small reagent addition. Two variations of this method were tested.

First Method – Karki_1: A slurry of 0.5 g of dried alga and 10 mL of DI water is sonicated for 2 minutes. More DI water is added at a volume of 10 mL to assure dispersion of the alga. The pH of the solutions is adjusted to 8.5 by adding 2 N NaOH dropwise (~ 35 μL). After vortexing, the tubes are then placed in a water bath at 60°C for 30 minutes. The samples are centrifuged to separate the alga from the aqueous phase.

Second Method – Karki_2: This method is the same as described above with the omittance of NaOH in the solution. This variation was used to assess the effect of pH adjustment on protein removal.

5.2.3 Multiple Extractions at High pH

Another batch of extractions was developed from methods described by Rausch. This paper addresses extraction from multiple classes of algae; green, diatoms and blue-green (cyanobacteria). The three algal cultures discussed were, _Scenedesmus acutus_ (green), _Asterionella formosa_ (diatom), and _Synechococcus spec._ (blue-green, cyanobacteria). This work found optimal processing conditions for each species.

First Method – Rausch_1: This is the standard method discussed by Rausch. A slurry is prepared by adding 5 mL of 0.5 N NaOH to 0.5 g of alga, followed by vortexing. Based on author recommendation, sonication is not used for this method. A total of three extractions are performed. The first extraction occurs at 80°C for 10 minutes in a water
bath. After the samples are cooled, the supernatants are removed. After adding 5 mL of 0.5 N NaOH (before each consecutive extraction), the remaining algal pellets are heated for 10 minutes at 100°C twice for a total of three extractions.

Second Method – Rausch_2: The second method differs from the first only by the addition of 200 μL of 3% H₂O₂ before the first extraction. This was found by the author to have the largest impact in the extraction of protein from green alga, as extraction can be more difficult due to well-structured cellulosic walls surrounding each cell [38].

5.2.4 Water Extractions

Fleurence et al. provides several procedures for protein extraction from seaweeds. The species examined were marine species Ulva rigida and Ulva rotundata. They have similarities to Cladophora glomerata in that they are macroalgae of the same division, Chlorophyta, and class, Ulvophyceae. These procedures take place at room temperature, minimizing harmful effects from high temperatures to the alga proteins. Three of the procedures that were assessed used Tris HCl buffer (0.1 M, pH 7.5) for extraction but gave inconclusive results when calculating protein removal. Tris HCl buffer is a combination of tris(hydroxymethyl)aminomethane and hydrochloric acid. Residual Tris in samples contributed to nitrogen content in the sample due to the chemical structure of Tris HCl, C₄H₁₁NO₃ClH.

The source lists all methods in the format of EX_ (where _ is a numerical value 1-8). One of the protocols tested is from Venkataraman et al. For comparison to the original literature, the annotations are listed in the description of each method.

First Method – Fleurence_1 (EX1): A solution of 0.5 grams of algal powder and 10 mL of DI water is made. Algal cells are lysed by osmotic shock. The tube is vortexed
and placed into a shaker water bath at room temperature. The shaker bath is set to a low agitation speed to allow the samples to be gently stirred overnight.

Second Method – Fleurence_2 (EX5): A weight of 0.5 g of dried alga is suspended in 10 mL DI water. The slurries are gently mixed overnight in a shaker water bath at room temperature. The supernatants are removed after centrifugation. The algal pellets are treated with 10 mL of 0.1 M NaOH and 0.5% v/v β-mercaptoethanol followed by vortexing. The solutions are stirred gently at room temperature in a water bath for 1 hour.

5.2.5 Calculations

CHN analysis was completed for both initial algal stock and solid pellets post extraction. Since extractions are run in triplicate, the average and standard deviation of the calculated protein content is reported on an algal dry weight basis.

The amount of protein removed from the alga, %p_{extracted}, can be estimated by the following equation where %p_f is the final protein content of the extraction runs, and %p_i is the protein content of the initial, untreated alga.

$$\% p_{extracted} = \frac{p_i(100) - \left( p_f \times \frac{(1-p_i)(100)}{(1-p_f)} \right)}{p_i(100)} \times 100\%$$ (5-1)

These calculations assume no significant losses of other algal components (i.e. lipids, starch and cellulose) and indicate the estimated success of the extraction techniques.
5.3 Extraction Results and Discussion

It is important to note that the results represent an estimated amount of protein. There are other nitrogen containing constituents in algae such as nucleic acids, amines, glucosamides, and cell wall materials [39]. By using total nitrogen content for protein estimation by CHN elemental analysis, it is likely to be an overestimation. Future work will be done to determine a more exact measure of protein, such as an amino acid analysis, but for preliminary testing the current method gives enough information to determine the best procedure for protein removal.

The results from all extractions are shown in Table 5.1.
**Table 5.1:** Protein extraction results for analysis on *Cladophora glomerata*. Summaries of methods are provided for comparison purposes.

<table>
<thead>
<tr>
<th>Method</th>
<th>Method Parameters</th>
<th>Average Nitrogen Content, $w_p$</th>
<th>% Protein</th>
<th>% Protein Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgin Cladophora 2009</td>
<td>Initial feedstock, untreated</td>
<td>3.03 ± 0.21</td>
<td>13.4</td>
<td></td>
</tr>
<tr>
<td>Karki_1</td>
<td>Water + NaOH 30 min @ 60°C sonicated</td>
<td>2.87 ± 0.06</td>
<td>12.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Karki_2</td>
<td>Water 30 min @ 60°C sonicated</td>
<td>2.91 ± 0.09</td>
<td>12.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Virgin Cladophora 2010</td>
<td>Initial feedstock, untreated</td>
<td>2.09 ± 0.10</td>
<td>9.28</td>
<td></td>
</tr>
<tr>
<td>Rausch_1</td>
<td>NaOH 10 min @ 80°C 10 min @ 100°C</td>
<td>0.41 ± 0.03</td>
<td>1.82</td>
<td>80</td>
</tr>
<tr>
<td>Rausch_2</td>
<td>NaOH + H$_2$O$_2$ 10 min @ 80°C 10 min @ 100°C</td>
<td>0.28 ± 0.03</td>
<td>1.26</td>
<td>86</td>
</tr>
<tr>
<td>Fleurence_1</td>
<td>Water overnight @ room temp</td>
<td>2.14 ± 0.00</td>
<td>9.50</td>
<td>-2.4</td>
</tr>
<tr>
<td>Fleurence_2</td>
<td>Water overnight @ room temp NaOH + β-mercaptoethanol 60 min @ room temp</td>
<td>1.71 ± 0.04</td>
<td>7.58</td>
<td>18</td>
</tr>
</tbody>
</table>

The multiple extractions, high pH procedures from Rausch, were quite successful with 80 and 86% protein removal. The two procedures are not overly complicated and time consuming, but do require multiple steps. This leaves room for varying the parameters to achieve maximum extraction. The effects of NaOH and H$_2$O$_2$...
concentrations, number of extractions, temperature and time of extractions, cell lysing techniques and washing the algal pellet can be investigated.

The chosen Raush_2 method will be implemented to test other received algal species to determine its versatility. Ideally, the optimized method will be usable on all algal species.

5.4 Preliminary Algal Hydrolysis Study

With promising results from protein extractions from *Cladophora glomerata*, experiments were formulated to determine if extraction exhibited charring of alga at hydrolysis conditions.

Trial reactor runs were performed using an apparatus constructed of Swagelok parts with a volume around 1mL.

![Figure 5-1: Swagelok assembly for algal runs at subcritical temperatures.](image)

This reactor is filled with an alga/water mixture. Typically, water is added to the alga in 15 mL tubes until the solids are suspended well enough to pour into the Swagelok
assembly. Once the assembly is charged with the algal solution, the ends are capped and it is submerged into a sand bath (Techne Fluidised Bath SBL-2) at 250°C.

Two lots of alga were used. The first was from the stock of untreated *Cladophora glomerata* that had not undergone any protein removal. The other was the final dried algal pellet from the protein extraction Rausch_2 that had removed 86% of the protein. Both lots began at a reaction time of 5 minutes. The time was incremented until charring was observed.

The following images show the results from the algal hydrolysis runs.

![Figure 5-2: Cladophora glomerata samples after testing at 250°C, a subcritical condition for water. (a) Untreated Cladophora, no protein extracted. (b) Cladophora after protein extraction procedure Rausch_2.](image)
There is a noticeable difference between the two lots of alga. The runs on untreated *Cladophora* show significant charring between 5-15 minutes. It is possible that charring could have happened early to these samples because they have not had protein extracted prior to hydrolysis conditions. Samples of *Cladophora* from the Rausch_2 procedure have shown to withstand high temperature between 60-90 minutes. This protein extracted alga still showed sign of degradation. This can be due to remaining 14% nitrogen or other components such as polysaccharides. Future testing will be designed to explore this phenomenon to achieve successful hydrolysis on algae.

5.5 Conclusions

Overall, the two methods for multiple extractions at high pH from Rausch had significant protein removal from *Cladophora glomerata*. These methods can now be used on different species to test the versatility. *Cladophora* does not have high lipid content and is therefore not the best candidate for hydrolysis runs, though it is easily collectable in large quantities. The two successful methods found from this early study will provide as a baseline method for manipulating processing parameters. Once a reliable protein removal process is established, alga can be introduced to the Parr reactor for studies on algal hydrolysis. Preliminary experiments of protein extracted alga at subcritical conditions showed improvement in stability.

CHN elemental analysis has provided a means to run multiple experiments and quickly gather quantitative extraction results. Experiments which show a considerable amount of nitrogen removal will be evaluated further with an amino acid analysis of proteins.
Chapter 6

Final Conclusions and Future Work

With increasing concern over the world’s fossil fuel supply and economic challenges, it is important to work towards the development of alternative energy sources. Algae show promise as an alternative biomass fuel source. Research to determine the logistics and technological challenges of using this biomass for biofuel production can have a major impact on the nation’s economy and independent fuel supply.

Efforts have been made to contribute to this significant research. In this study, focus was placed on optimizing processing conditions and characterizing algal species. Methods for converting algal lipids to biodiesel in order to determine fatty acid methyl ester (FAME) content have been compared. A two-step green processing technique to recover free fatty acids (FAs) from soybean oil has been studied and modeled as a precursor to algal hydrolysis. Characterization of algal species has been conducted to better understand the potential end-products, and lot-to-lot variation. Finally, protein extraction as a pre-processing strategy for algal hydrolysis was investigated to improve reaction conditions.

Lipid/FAME analysis is an important tool in determining the viability of an algal species as a feedstock for biofuel production. A direct, in situ, method, Chloroform
Direct Method (CDM), was found to produce comparable results to the Bligh and Dyer/Napolitano (B&D/Nap) method which requires wet samples. In the CDM, a dry algal sample is used, eliminating the variability of the dry weight of sample used in FAME content calculations. The direct conversion of lipids to FAMEs significantly decreases processing time, labor, and room for error. The CDM uses a smaller sample size, 20 mg, compared to B&D/Nap which uses ~100 – 200 mg dry algae. Supplemental experiments using 100 mg of starting material were performed for the CDM. There was slight variability in FAME content. Statistically, it was found that using 100 mg was not significantly different from the B&D/Nap but 20 mg was significantly different. It is suggested to investigate this further by varying sample size for the CDM to determine the significance on the FAME content results and comparability to the B&D/Nap.

A kinetic model for batch soybean oil hydrolysis has been developed after studying agitation speed and temperature effects on the yield of FAs as a function of time. The model agrees well with experimental data and will be used as a platform for algal hydrolysis. The model can be used as a tool to predict reaction behavior for an algal system and to test the autocatalytic behavior of FAs. Further soybean oil experiments spiked with recycled FA product from previous runs, or an external source of FA, at the start of the reaction could exhibit faster reaction rates and FA yield. This would decrease the reaction time. The shorter reaction time could be beneficial for energy savings and avoiding the degradation of alga at prolonged exposure to high temperatures. The model can be tested for robustness by application to algal hydrolysis. Algal hydrolysis is expected to be more complicated than soybean oil hydrolysis. Some likely challenges are obtaining sufficient algal lipid content to ensure effective hydrolysis, difficult clean-up of
the Parr reactor components, charring of other algal constituents at high temperatures decreasing lipid accessibility, and meeting energy requirements for full algal processing to achieve a positive energy gain. These issues are important to address when transitioning from the hydrolysis of soybean oil to algae.

The characterization of lipids, carbohydrates and protein contained in algae is essential in determining the usefulness of each species. Processing techniques to isolate these constituents are necessary to develop methods to use entire lots of algae and optimize the economics of operation. Each biopolymer is useful and could be processed to obtain multiple consumer products. Completing a mass balance on algal samples is recommended to determine the three main biopolymers and other constituents. Additional analytical techniques may be needed to entirely close an algal mass balance.

Algal hydrolysis will provide a green processing technique to convert lipids to FAs, which can then be used to produce multiple end-products. Algae are complex organisms with potential to char and degrade under hot water conditions. Again, it is important to use as much of the algal biomass as possible to receive a positive economic return. If valuable constituents are damaged during hydrolysis, they will no longer be usable. Preliminary experiments have shown that the presence of protein in algal samples at subcritical conditions results in charring at early stages of the reaction. Protein extraction has been addressed in this study. A promising extraction technique has shown over 80% protein removal from green algae. Optimization of this extraction method is necessary. Multiple parameters can be adjusted to increase extraction. It would be ideal to determine a universal extraction technique to be used on all algal species. This will require testing an optimized method on various species to determine its versatility. A
more rigorous amino analysis for exact protein measurement is also suggested. An in depth study of carbohydrate content before and after hydrolysis, as well as the effects on the reaction is also recommended.

On a final note, it would be useful for all researchers and developers if a completed life cycle assessment (LCA) is developed for fuels and chemicals derived from algal feedstocks. Algal systems are highly complex, from the growth of algae to finding the most economical and efficient processing techniques. With the large variability of the distribution of biopolymers between algal species, the available product and quantities can be inconsistent. All studies performed on algae including algal growth, characterization, and processing are important stepping stones in developing a useful LCA. Determining the environmental and economic benefits of algae to fuel will open the opportunities to turn bench scale studies to a commercial reality.
References


Appendix A

Kinetic Model: Concentration Profiles of Soybean Oil Hydrolysis Components

This Appendix contains concentration information regarding all hydrolysis components; triglycerides (TG), diglycerides (DG), monoglycerides (MG), glycerol (G), free fatty acids (FA) and water (W). Initial concentrations of the mole species balances in equations 3-15 to 3-20 are shown in Figure A-1. The ordinary differential equations were solved using a fourth-order Runge-Kutta method (equation 3-27) to predict the time course of the hydrolysis reaction. Plots of the predicted individual component concentrations and the experimental FA concentration data (FA exp) versus time for four reaction temperatures (225, 250, 275 and 300°C) are shown in Figures A-2 through A-5.

Table A.1: Initial concentrations of hydrolysis components and theoretical free fatty acid yield. ($MW =$ molecular weight, $C_i =$ concentration)

<table>
<thead>
<tr>
<th>Component</th>
<th>$MW$ (g/mol)</th>
<th>Weight (g)</th>
<th>$C_i$ (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>871</td>
<td>30</td>
<td>2.26E-01</td>
</tr>
<tr>
<td>DG</td>
<td>611</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MG</td>
<td>352</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>92</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FA</td>
<td>278</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>W</td>
<td>18</td>
<td>120</td>
<td>4.37E+01</td>
</tr>
</tbody>
</table>

Theoretical FA (mol/L) 6.77E-01
Figure A-1: Concentrations of hydrolysis species versus time at 225°C.

Figure A-2: Concentrations of hydrolysis species versus time at 250°C.
Figure A-3: Concentrations of hydrolysis species versus time at 275°C.

Figure A-4: Concentrations of hydrolysis species versus time at 300°C.